

Further Purification and Characterization of a Multienzyme Complex for DNA Synthesis in Human Cells

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Abstract The 21 S complex of enzymes for DNA synthesis in the combined low salt nuclear extract-post microsomal supernatant from HeLa cells [Malkas et al. (1990) *Biochemistry* 29:6362–6374] was purified by poly (ethylene glycol) precipitation, Q-Sepharose chromatography, Mono Q Fast Protein Liquid Chromatography (FPLC), and velocity gradient centrifugation. The procedure gives purified enzyme complex at a yield of 45%. The 21 S enzyme complex remains intact and functional in the replication of simian virus 40 DNA throughout the purification. Sedimentation analysis showed that the 21 S enzyme complex exists in the crude HeLa cell extract and that simian virus 40 in vitro DNA replication activity in the cell extract resides exclusively with the 21 S complex. The results of enzyme and immunological analysis indicate that DNA polymerase α -primase, a 3',5' exonuclease, DNA ligase I, RNase H, and topoisomerase I are associated with the purified enzyme complex. Denaturing polyacrylamide gel electrophoresis of the purified enzyme complex showed the presence of about 30 polypeptides in the size range of 300 to 15 kDa. Immunofluorescent imaging analysis, with antibodies to DNA polymerase α , β and DNA ligase I, showed that polymerase α and DNA ligase I are localized to granular-like foci within the nucleus during S-phase. In contrast, DNA polymerase β , which is not associated with the 21 S complex, is diffusely distributed throughout the nucleoplasm. © 1993 Wiley-Liss, Inc.

Key words: DNA replication, multienzyme complex, HeLa cells, SV40, enzymes

Chromosomal DNA replication is a complex process that requires many protein-protein and protein-DNA interactions that must occur with high precision [Echols, 1986]. In prokaryotes precision of DNA replication occurs through the organization of many enzymes and nonenzymic proteins into multienzyme complexes [Alberts and Miake-Lye, 1985; Kornberg and Baker, 1992]. Chromosomal DNA replication in eukaryotes must occur with equal or greater precision than it does in prokaryotes. There have been reports of multienzyme complexes for DNA replication in eukaryotes [Tubo and Berezney, 1987a; Jackson and Cook, 1986a; Jazwinski and Edelman, 1984; Reddy and Pardee, 1980]. There is little information available, however, on the

functional organization of the DNA synthesizing machinery in eukaryotic cells.

Simian virus 40 (SV40) has attributes that make it tractable for the investigation of the replication machinery in primate cells [reviewed in Challberg and Kelly, 1989; Stillman, 1989]. The initiation of SV40 DNA replication occurs at a single replication origin (ori) and, with the exception of the viral encoded large T-antigen (T-ag), requires the primate host cell's DNA synthesizing machinery for replication. A cell free system was developed for SV40 ori- and T-ag-dependent replication of SV40 DNA in vitro [Li and Kelly, 1984]. This system uses the DNA synthesizing apparatus present in a cell free extract from primate cells supplemented with purified T-ag and efficiently replicates with supercoiling exogenous plasmid templates containing the minimal SV40 ori [Li and Kelly, 1984; Stillman and Gluzman, 1985; Wobbe et al., 1985; Yamaguchi and DePamphilis, 1986]. The use of the cell-free system for SV40 DNA replication in vitro in fractionation/reconstitution studies of cell extracts has identified several host cell pro-

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teins required for SV40 DNA replication in vitro [reviewed in Challberg and Kelly, 1989; Stillman, 1989].

We previously showed that T-ag dependent SV40 in vitro replication activity in HeLa cell extracts resides with a soluble sedimentable, 21 S complex. The 21 S complex contains requisite enzymes and nonenzymic proteins for DNA synthesis [Baril et al., 1988; Hickey et al., 1988; Malkas et al., 1990a]. The efficiency and requirements for the replication of SV40 DNA in vitro by the isolated enzyme complex are comparable to those found for SV40 DNA replication in vitro using crude cell extracts [Malkas et al., 1990a]. In this paper we report on the purification of the 21 S enzyme and show that the complex exists in crude cell extracts. We also show by immunofluorescent analysis that enzymes associated with this 21 S enzyme complex are localized with granular-like structures within the nucleus.

MATERIALS AND METHODS

Materials

PSVO⁺ plasmid DNA, containing the HindIII-SphI SV40 ori DNA fragment [Stillman and Gluzman, 1985], and immunoaffinity purified SV40 T antigen from extracts of HeLa cells infected with the adenovirus recombinant R284 were prepared according to a published procedure [Wold et al., 1989]. Hybridoma cell lines secreting monoclonal antibodies SJK 132-20 and SJK 237-71 to human DNA polymerase α [Tanaka et al., 1982] were obtained from ATCC. The secreted antibodies (IgG fraction) were purified as described [Malkas et al., 1990a]. Rabbit polyclonal antibody to calf thymus DNA ligase I [Tomkinson et al., 1990] was a gift from Dr. Tomas Lindahl of the Imperial Cancer Research Fund, U.K. Q-Sepharose, Mono Q HR5/5, HR10/10 columns, and the FPLC system were purchased from Pharmacia LKB Biotechnology, Inc. Electrophoresis reagents were obtained from Bio-Rad Laboratories. All other reagents that were used have been described in publications from this laboratory [Malkas and Baril, 1989; Malkas et al., 1990a; Vishwanatha and Baril, 1990].

Methods

Cell growth, synchronization, and subcellular fractionation. HeLa S₃ cells were grown in suspension cultures and exponentially growing cells were harvested as previously described [Lamothe et al., 1981]. Cell synchroniza-

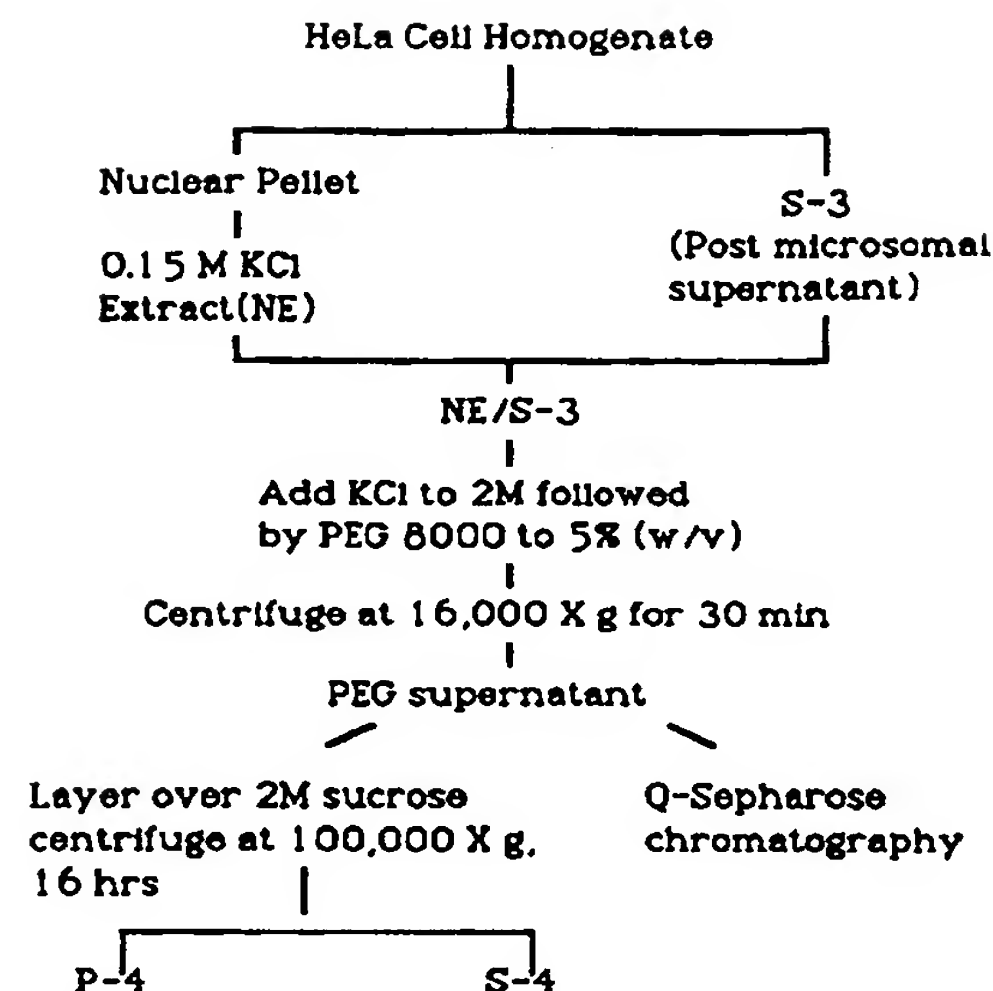


Fig. 1. Flow diagram of the initial steps in the procedure for purification of the 21 S enzyme complex that functions in *in vitro* SV40 DNA replication. Homogenates were routinely prepared from 20–40 g (wet weight) of HeLa cells. Details of the purification procedure are given under Materials and Methods.

tion was by the double-thymidine block technique [Chiu and Baril, 1975]. HeLa cell homogenates were subfractionated by a published procedure [Malkas et al., 1990a] that is outlined in Figure 1. Briefly, nuclei and the postmicrosomal supernatant solution (S-3) were isolated from a 30% HeLa cell homogenate and the nuclei were suspended in 4 vol of 50 mM Tris-HCl [Tris (hydroxymethyl)aminomethane] pH 7.5, 1 mM EDTA-Na₃ (ethylenediaminetetraacetic acid, pH 7.5), 1 mM EGTA-Na₃ (ethylene[bis(oxyethylenenitrilo)]tetraacetic acid, pH 7.5) (buffer A) containing 1 mM aminoacetoneitrile (AAN), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.15 M KCl. The suspension was rocked at 4°C for 1 h, followed by centrifugation at 10,000g for 10 min. The resulting supernatant (nuclear extract, NE) was combined with the S-3 and the combined nuclear extract/postmicrosomal supernatant (NE/S-3) was used directly for purification of the enzyme complex or first subjected to poly[ethylene glycol] (PEG) precipitation. For PEG precipitation the NE/S-3 was adjusted to 2 M KCl and 5% PEG 8000 concentration with rocking at 4°C for 1 h. After centrifugation at 16,000g for 30 min, essentially all the SV40 in vitro replication activity is recovered in the resulting supernatant (PS) [Malkas

et al., 1990a]. The PS fraction was dialyzed against buffer-A containing 10% glycerol, 1 mM AAN, 1 mM PMSF, 0.15 M KCl, and used directly for Q-Sepharose chromatography or Mono Q FPLC or first subjected to discontinuous gradient centrifugation as outlined in Figure 1.

Discontinuous gradient centrifugation. The PS was layered onto a 2 M sucrose cushion in buffer A and centrifuged at 100,000g for 16 h at 4°C. Following centrifugation the supernatant solution (S-4 fraction) and the 2 M sucrose interphase (P-4 fraction) were successively removed by aspiration and dialyzed against 50 mM Tris-HCl, pH 7.5, 0.15 M KCl, 1 mM EDTA- Na_3 , and 10% glycerol. In a previous study we showed that discontinuous gradient centrifugation of the PS under these conditions separates a sedimentable P-4 from a nonsedimentable S-4 fraction of enzymes for DNA synthesis [Baril et al., 1988; Hickey et al., 1988; Malkas et al., 1990a,b]. The P-4 was subjected to Mono Q FPLC for purification of the sedimentable complex of enzymes for DNA synthesis.

Q-Sepharose chromatography. Chromatography on Q-Sepharose was done either by conventional column chromatography [Malkas et al., 1990a,b] or FPLC on a HR 10/10 column with essentially the same results. For conventional Q-Sepharose chromatography, the PS was dialyzed against buffer-A containing 1 mM AAN, 1 mM PMSF, and 0.15 M KCl and loaded onto a Q-Sepharose column (1 cm^3 bed volume/25 mg protein) equilibrated with the dialysis buffer. After washing with 8 column volumes of the equilibration buffer, the column was eluted with 10 column volumes of a continuous gradient of increasing KCl concentration from 0.15 to 1 M in buffer-A. Fractions of 1 to 2 mL were collected at a flow rate of 0.5 mL/min, dialyzed for 12 h against buffer-A containing 1 mM AAN, 1 mM PMSF, 1 mM dithiothreitol (DTT), and 0.15 M KCl, and assayed for protein and the respective enzyme activities. The peak fractions of the eluted enzyme activities from Q-Sepharose were pooled and used immediately for further purification or stored at -80°C.

We previously showed that during Q-Sepharose chromatography of the PS, NE/S-3, or the P-4 fractions, essentially all the SV40 in vitro replication activity and the 21 S enzyme complex for DNA synthesis bind to the matrix and are eluted between 0.15 M to 0.25 M KCl concentrations while 30% to 50% of the loaded protein does not bind and appears in the column flow-

through [Baril et al., 1988; Hickey et al., 1988]. There is no detectable SV40 in vitro replication activity in the column flow-through fraction.

Mono Q FPLC of the enzyme complex for DNA synthesis. Aliquots (usually 10–20 mg protein) of the NE/S-3, PS, P-4, or the Q-Sepharose eluted fraction from partial purification of the enzyme complex (Fig. 1) were analyzed by FPLC on a Mono Q HR 5/5 column equilibrated with buffer-B (20 mM Tris-HCl, pH 8.3, 1 mM EDTA- Na_3 , 1 mM EGTA- Na_3 , and 1 mM DTT) containing 0.15 M KCl. The sample was dialyzed for 2 h against buffer-B containing 1 mM AAN plus 1 mM PMSF and injected on to the column at a low flow rate (0.2 mL/min). After loading, the column was washed with 10 mL buffer-B containing 0.15 M KCl and eluted with a decreasing pH gradient from 8.3 to 6.2 composed of 0–100% buffer-C (20 mM Bis Tris pH 6.2, 1 mM EDTA- Na_3 , 1 mM EGTA- Na_3 , and 1 mM DTT) containing 0.15 M KCl. The back pressure of the column was maintained below 200 psi throughout the run. The individual enzyme and SV40 replication activities associated with the enzyme complex eluted as a single peak following the injection of 2 mL of buffer-C containing 0.5 M KCl. Fractions containing the peak of the eluted enzyme and SV40 replication activities were pooled, dialyzed against buffer-A containing 1 mM DTT, 1 mM AAN, 1 mM PMSF, 0.15 M KCl, and 10% glycerol, and were used immediately or stored at -80°C.

Velocity gradient centrifugation. The enzyme complex was sedimented on preformed 10–35% glycerol gradients prepared in buffer-A containing 0.15 M or 0.5 M KCl and formed in polyallomer tubes for the Beckman SW50.1 rotor as described previously [Malkas et al., 1990a]. A 0.1 mL sample (about 0.2 mg protein) was layered on top of the gradient and centrifuged at 196,000g for 16 h at 4°C. Sedimentation markers that included porcine thyroglobulin (19 S), horse spleen apoferritin (17 S), and yeast alcohol dehydrogenase (7 S) were run on parallel gradients. After centrifugation, 0.15 mL fractions were collected by tube puncture from the bottom and assayed for the respective enzyme and SV40 replication activities. Sedimentation values were calculated according to the procedure of Siegel and Monty [1966].

Enzyme assays. The activities of DNA polymerase α [Lamothe et al., 1981], DNA primase [Vishwanatha and Baril, 1986], 3',5' exonuclease [Skarnes et al., 1986], DNA-dependent

ATPase [Vishwanatha and Baril, 1990], DNA ligase [Hickey et al., 1988], and RNase H activity [Baril et al., 1988] were assayed according to published procedures from this laboratory. DNA polymerase δ activity was assayed according to a published procedure [Tan et al., 1986]. Type I topoisomerase activity was assayed for the relaxation of pUC8 DNA according to the procedure of Liu and Miller [1981]. Type II topoisomerase activity was assayed for the unknotting of *Cri-thidia fasciculata* kinetoplast DNA using the procedure of Miller et al. [1981]. One unit of DNA polymerase α activity equals 1 nmol of total dNMP incorporated into DNA per h at 35°C. One unit of DNA primase activity equals 1 nmol of NMP incorporated into acid-insoluble material per h at 30°C. One unit of exonuclease activity equals the hydrolysis of 10 nmol of total dNMP to acid-soluble form in 30 min at 35°C. One unit of ATPase activity equals the hydrolysis of 1 nmol of ATP per h at 30°C. One unit of RNase-H activity equals 1 nmol of [3 H]poly(A) converted to acid-soluble form per h at 30°C.

SV40 in vitro replication. In vitro replication of pSV0⁺ plasmid DNA was assayed according to the procedure of Wold et al. [1989]. One unit of SV40 replication activity is designated as the incorporation of 2 pmol of dNMP into DpnI resistant SV40 DNA per h under the standard assay condition.

For analysis of the replication product the DNA was isolated by phenol/chloroform extraction followed by precipitation with isopropanol in the presence of 2 M ammonium acetate. The isolated DNA was resuspended in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA- Na_3 and digested with 8 units of DpnI to remove the unreplicated plasmid DNA [Malkas et al., 1990a]. Samples of the DpnI resistant in vitro replicated DNA product were electrophoresed on 1.5% agarose gels in TBE buffer (89 mM Tris-borate, 89 mM boric acid, and 1 mM EDTA- Na_3 , pH 7.8).

Polyacrylamide gel electrophoretic analysis of proteins. Polyacrylamide gel electrophoretic (PAGE) analysis of proteins under nondenaturing and denaturing conditions was as described [Lamothe et al., 1981; Vishwanatha et al., 1986]. The protein stained bands were developed by Coomassie blue or silver staining.

Western blot analysis. Protein samples were electrophoresed under nondenaturing or denaturing conditions according to published procedures [Lamothe et al., 1981; Vishwanatha

et al., 1986]. Electrophoretic transfer of the separated proteins was according to the procedure of Towbin and co-workers [1979] with modifications described previously [Malkas et al., 1990a].

Immunofluorescent microscopy and image processing. Synchronized HeLa cells were seeded under aseptic conditions at a cell density of $5 \times 10^4/\text{mL}$ on coverslips, pretreated with E-C-L cell attachment matrix, in 60 mm culture dishes (LUX). After washing with phosphate-buffered saline (PBS) the cells were fixed in freshly prepared 4% paraformaldehyde-PBS for 10 min, rinsed twice for 5 min in PBS containing 0.2% Triton X-100. The cover slips were soaked for 30 min in PBS containing 20% fetal calf serum (Hyclone, Salt Lake City, UT) for blocking. This was followed by incubation of the fixed-permeabilized cells for 1 h with appropriate dilutions of the purified primary antibody IgG (1:50 dilution for rabbit anti-calf thymus DNA ligase I and 1:250 dilutions of mouse anti-human polymerase α and anti-DNA polymerase β recombinant protein, respectively) followed by extensive washing with PBS. This was followed by a 1 h incubation with the appropriate fluorescein conjugated secondary antibody, goat anti-mouse IgG, or goat anti-rabbit IgG (Boehringer Mannheim Biochemicals) diluted 1:250 in PBS followed by extensive washing with PBS. All the above procedures were done at room temperature.

Immunofluorescent images were obtained with a Zeiss IM 35 inverted microscope (Carl Zeiss Inc., Thornwood, NY) equipped with a 100 X/NA 1.30 Neofluor objective a 63X/NA 1.25 Neofluor objective and a low-light level ISIT Video camera (Dage-MTI, Inc., Michigan City, IN). Fluorescence was detected with the Zeiss 487717 filter set. A 100 W quartz-halogen lamp, operated below 7 V, was used as the light source. Raw images from the ISIT camera were processed with an image processing system (Series 150, Imaging Technology, Woburn, MA), by summing 300 frames and subtracting the corresponding dark images. The resulting images were then scaled to obtain pixel intensity values between 0 and 255.

Other methods. Neutralization of DNA polymerase α and SV40 in vitro replication activity were done as previously described [Malkas et al., 1990a]. Protein was determined by the Bradford procedure [1976] using bovine serum albumin (BSA) as the standard.

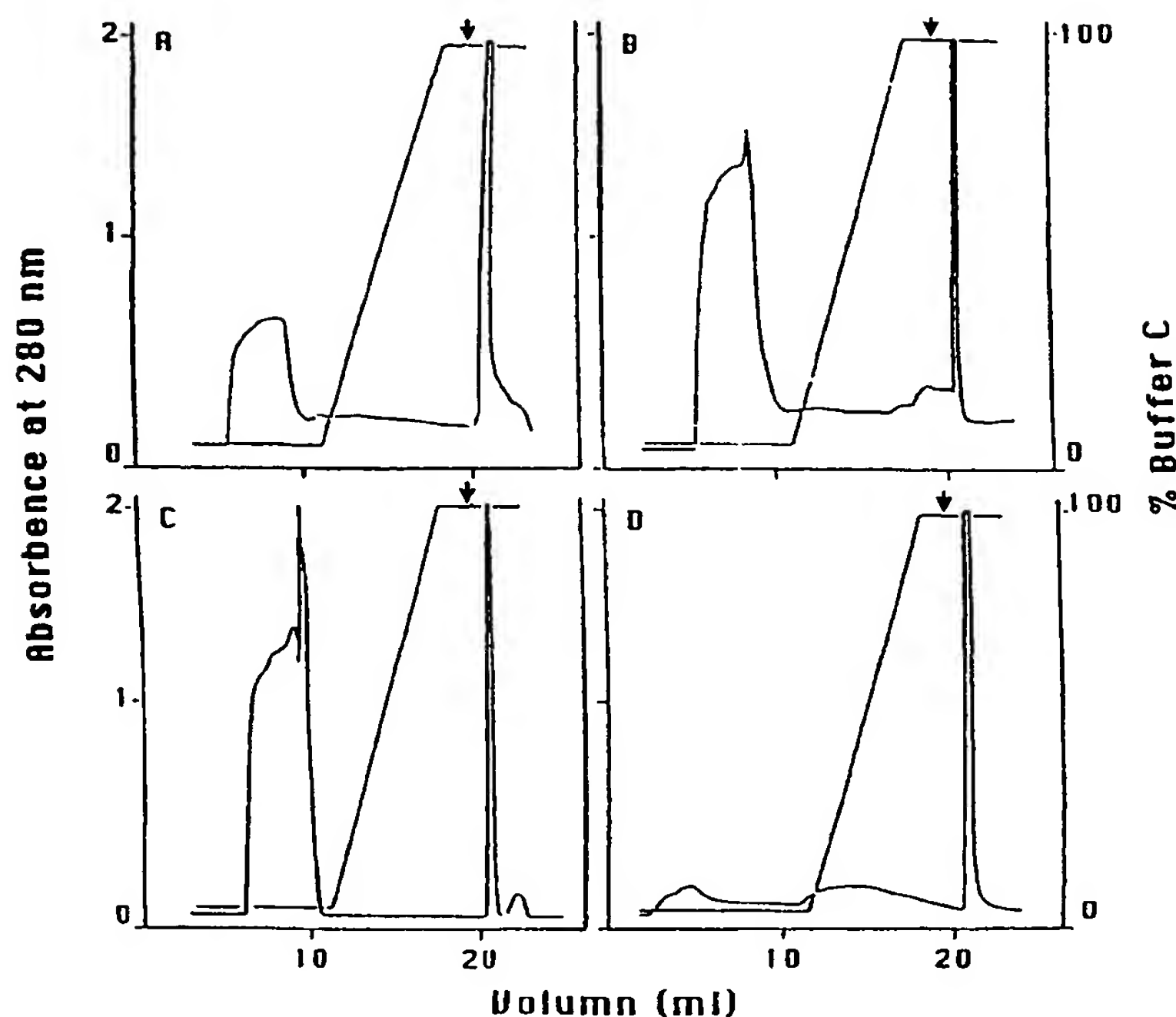


Fig. 2. Elution profiles of 280 nm absorbing material from Mono Q FPLC of intermediate fractions in the purification of the 21 S enzyme complex. The NE/S-3, PS, P-4 and the peak of enzyme, and SV40 DNA replication activity eluted from Q-Sepharose chromatography of the PS (Fig. 1) were analyzed by FPLC on a Mono Q (HR 5/5) column. Elution was by an inverse pH gradient from 8.3 to 6.2 followed by an increase in KCl

concentration to 0.5 M in buffer-C as described under Materials and Methods. Mono Q FPLC of (A) 21 mg of protein from the NE/S-3, (B) 19 mg of protein from the PEG supt, (C) 17 mg of protein from the P-4 fraction, and (D) 18 mg of protein from the peak of enzyme and SV40 DNA replication activity eluted from Q-Sepharose of the PS. Injection of 2 mL buffer C containing 0.5 M KCl indicated by arrow.

RESULTS

The *in vitro* SV40 DNA replication activity in the combined NE/S-3 fraction from HeLa cells resides exclusively with a soluble, but sedimentable, complex that contains essential enzymes for DNA synthesis [Baril et al., 1988; Hickey et al., 1988; Malkas et al., 1990a,b]. To help understand the nature of the complex of enzymes for DNA synthesis and to assist in defining its function, the complex was purified from different fractions of the cell homogenate.

Mono Q FPLC of the 21 S Complex

The initial steps in the purification of the 21 S enzyme complex were according to a published procedure [Malkas et al., 1990a] outlined in Figure 1 with modifications described under Experimental Procedures. Typical elution profiles

of the 21 S enzyme complex during Mono Q FPLC of the crude NE/S-3 fraction and intermediate fractions during the purification are shown in Figure 2A–D. Most of the 280 nm absorbing material in the NE/S-3, P-4, and PS fractions did not bind to the Mono Q column and was recovered in the column flow-through/wash (Fig. 2A–C). The increased amount of 280 nm absorbing material recovered in the column flow through for the PS and P-4 fractions is due to residual PEG in these fractions that does not bind to the anion exchanger. Since 50% of the protein in the PS fraction does not bind to Q-Sepharose [Malkas et al., 1990b], the amount of 280 nm absorbing material in the column flow-through/wash was greatly reduced during Mono Q FPLC of the 21 S enzyme complex eluted from Q-Sepharose (compare Fig. 2B and 2D). Some 280 nm absorbing material was eluted

from the Mono Q column by the inverse pH gradient from 8.3 to 6.2. A single, sharp peak of protein was eluted from the Mono Q column by the abrupt increase in the KCl concentration to 0.5 M following the inverse pH gradient. This eluted peak, which represents only about 5% of the loaded protein, was present in the Mono Q FPLC elution profile for each of the four starting fractions (Fig. 2A–D).

Enzymes for DNA Synthesis and SV40 Replication Activity Coelute During Mono Q FPLC

The recovery of enzymes for DNA synthesis and in vitro SV40 DNA replication activity during Mono Q FPLC is exemplified in Figure 3A,B for the recovery of DNA polymerase α and SV40 DNA replication activities in the NE/S-3 and the eluted 21 S complex from Q-Sepharose chromatography of the PS (shown in Fig. 2A and 2D, respectively). Similar recoveries of these activities were also observed during Mono Q FPLC of the PS and P-4 (data not shown). A small amount of DNA polymerase activity that functions with activated but not primed, ss DNA templates was recovered in the column flow-through/buffer-A wash fraction during Mono Q FPLC of the NE/S-3 fraction (Fig. 3A). This is due to nonsedimentable DNA polymerase α -primase and activities of other enzymes for DNA synthesis that were shown previously not to be associated with 21 S complex. These activities are recovered in the nonsedimentable S-4 fraction during discontinuous gradient centrifugation of the PS [Baril et al., 1988; Hickey et al., 1988]. No DNA polymerase α activity was recovered in the column flow-through/buffer-A wash fraction during Mono Q FPLC of the sedimentable P-4 (data not shown) or the eluted fraction containing the 21 S enzyme complex from Q-Sepharose chromatography of the PS (Fig. 3B). Also, no SV40 DNA replication activity, or the activities of other enzymes for DNA synthesis (data not shown), was recovered in the column flow-through/buffer-A wash fraction or fractions eluted by the inverse pH gradient during Mono Q FPLC of each of the four starting fractions (Fig. 3A,B and data not shown). More than 90% of the loaded SV40 DNA replication activity, DNA polymerase α and activities of the other enzymes for DNA synthesis were routinely recovered from the Mono Q column with the sharp peak of 280 nm absorbing material that was eluted following the inverse pH gradient by the step of the

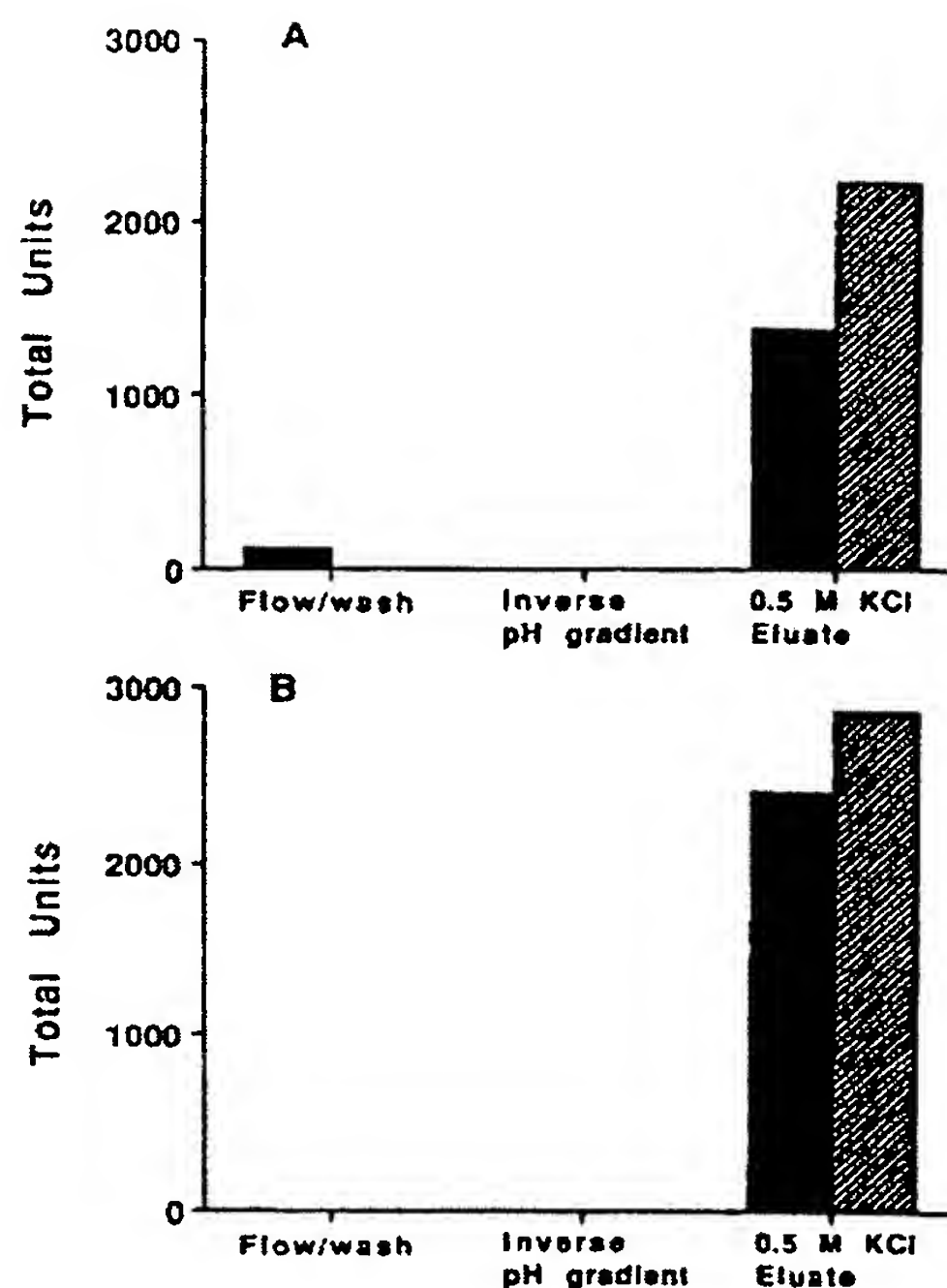


Fig. 3. Recovery of DNA polymerase α and in vitro SV40 DNA replication activities from Mono Q FPLC of the NE/S-3 and the peak of enzyme and SV40 DNA replication activity eluted from Q-Sepharose chromatography of the PS. Recovery from Mono Q FPLC of (A) the NE/S-3 (Fig. 2A) and (B) the peak of activity eluted from Q-Sepharose of the PS. Solid column, DNA polymerase α activity; Hatched column, in vitro SV40 DNA replication activity. Chromatographic conditions were as described in the legend to Figure 2 and under Materials and Methods.

KCl concentration to 0.5 M (Fig. 3A,B and data not shown).

Table I summarizes some activities of enzymes for DNA synthesis that were recovered in the 0.5 M KCl eluted peak from the Mono Q column. Although this table depicts recoveries of enzyme activities from Mono Q FPLC of the Q-Sepharose eluted fraction (Fig. 2D), similar recovery of enzyme activities were also observed during Mono Q FPLC of the NE/S-3, P-4, and PS. Figure 4A,B shows that this peak also contains topoisomerase I and DNA ligase activities. The purified ligase has the physical and enzymatic properties of DNA ligase I (Li and Baril, unpublished data). The 3' to 5' exonuclease activity recovered with this fraction is specific for ss DNA substrates and has the properties of the exonuclease that was previously reported to cofractionate with the 640 kDa multiprotein

TABLE I. Enzyme Activities Associated With the Purified 21 S Complex of Enzymes for DNA Synthesis*

Enzyme activity	Total units ^a
DNA polymerase with:	
activated DNA	1,810
primed ss-DNA	1,363
DNA primase	471
DNA-dependent ATPase	3,665
3' to 5' exonuclease	115
RNase-H	2,214

*The purification was from a homogenate of 20 g of HeLa cells. Total units are for one half of the eluted 21 S complex from Q-Sepharose chromatography of the PS that was subjected to Mono Q FPLC.

^aUnits are defined under Materials and Methods.

DNA polymerase α -primase complex [Skarnes et al., 1986]. Some exo- and endo-nuclease, DNA ligase, and RNase H activities were also recovered in the column flow-through/buffer-A wash during Mono Q FPLC of the NE/S-3 and PS. This is attributable to a segment of the activities of these enzymes in the crude NE/S-3 and PS fractions that is not associated with sedimentable complex of enzymes for DNA synthesis [Baril et al., 1988; Malkas et al., 1990b].

SV40 in vitro replication activity was exclusively recovered with the sharp, peak of enzyme activities eluted from the Mono Q column by the abrupt increase in KCl concentration to 0.5 M following the inverse pH gradient (Fig. 3A,B). The result from gel analysis of the products synthesized by this fraction during a 3 h incubation for the in vitro SV40 DNA replication assay conducted in the presence or absence of T-ag is shown in Figure 5. Synthesis occurred after a 15 min lag period with the generation mainly of Form II (relaxed, open circles) DNA (Fig. 5 left panel). The synthesis of topological intermediates, Form I (supercoiled, closed circles), Form II DNA, and high molecular weight DNA occurred during 30 to 60 min of the incubation. The synthesis of these products increased during the remainder of the 3 h incubation. The presence of replication intermediates or products from abortive replication that migrate between Form II and the high molecular weight DNA was not apparent in the gel analysis of the products synthesized throughout the 3 h incubation (Fig. 5, left panel). In agreement with our analysis with a less pure enzyme preparation [Malkas et al., 1990a,b], SV40 replication by the purified enzyme complex was completely depen-

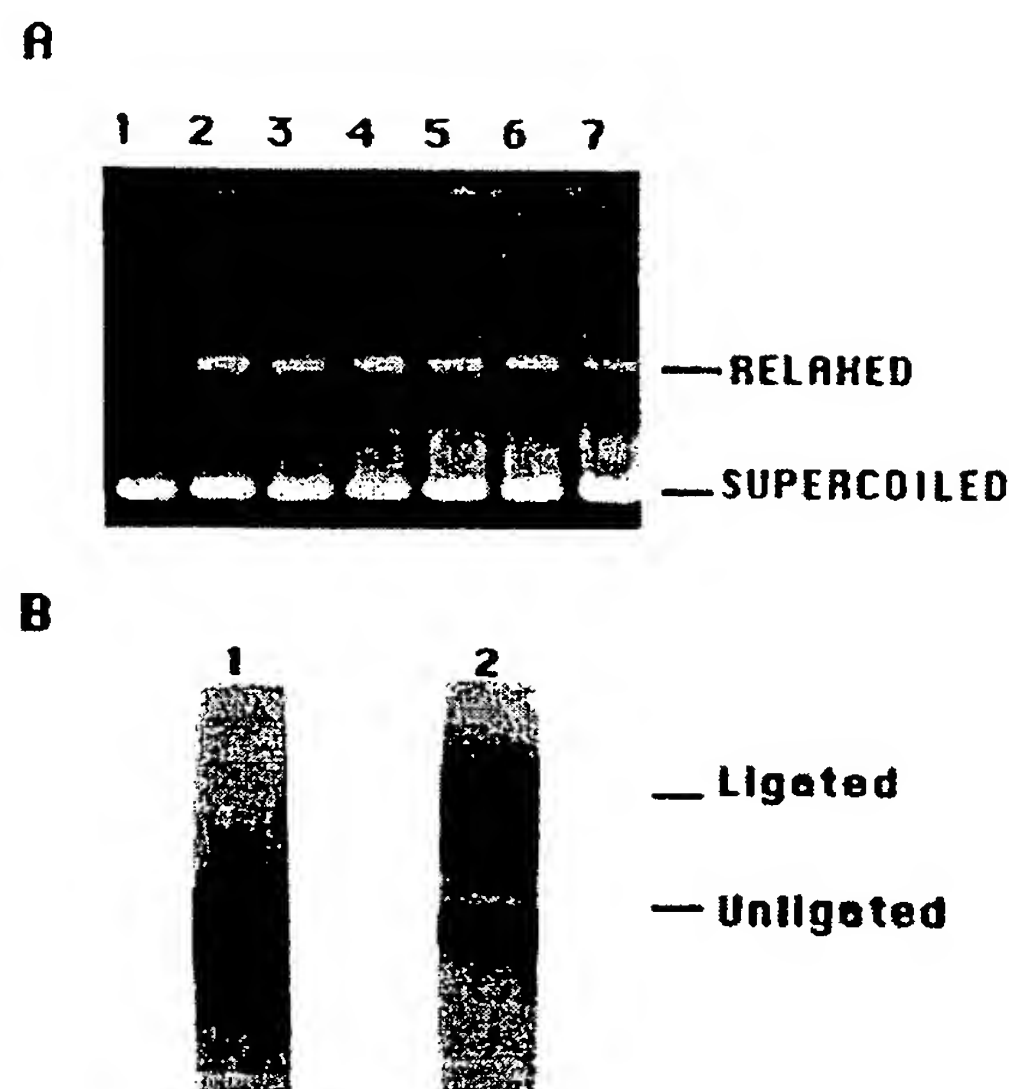


Fig. 4. Topoisomerase I and DNA ligase activities in the 0.5 M KCl/buffer-C eluted peak from Mono Q FPLC of the peak of enzyme and in vitro SV40 DNA replication activity from Q-Sepharose of the PS. A: Topoisomerase I activity was assayed with pUC8-14 DNA as substrate. Assays were performed with varying amounts of protein (0 to 7.5 μ g) from the 0.5 M KCl/buffer-C eluted peak from Mono Q. Incubation was at 35°C for 20 min and the gel analysis was performed according to Liu and Miller [1981]. Lanes 1-(0), 2-(1), 3-(2), 4-(3), 5-(4), 6-(5), and 7-(7.5) are from gel analysis of the topoisomerase I assays in which the designated (μ g) amounts of protein were used. B: DNA ligase activity was assayed according to a published procedure [Hickey et al., 1988] using as substrate a 20 mer oligodeoxynucleotide (5' CCGCCTCTGATTCCAGAAGT 3') annealed with two complimentary 10 mer oligodeoxynucleotides (3' GCCGGAGACT* 5') and (3' AAGGTCTTCA 5') in which one 10mer was 5' end labeled (*) with 32 P. The substrate represents sequences in the ori region of SV40 DNA between nucleotides 5215 to 5239. The reaction contained 50 mM Tris-HCl, pH 7.8, 10 mM MgCl₂, 2 mM ATP, 10 mM DTT, 2.5 μ g oligonucleotide, and 5 to 15 μ g of enzyme protein. Incubation was at 30°C for 60 min and the ligated product was analyzed by electrophoresis on a 15% polyacrylamide gel in the presence of 8 M urea. Lane 1, control (minus enzyme); Lane 2, 10 μ g protein of the 0.5 M KCl/buffer-C eluted peak containing the enzyme and SV40 DNA replication activity from the Mono Q column.

dent on the presence of T-ag and plasmid DNA containing functional SV40 minimal replication origin sequences in the reaction (Fig. 5, right panel and data not shown). Also, the majority of the products were resistant to DpnI digestion but were linearized by Sall digestion (data not shown), suggesting that they resulted from semi-conservative replication [Li and Kelly, 1984; Stillman and Gluzman, 1985].

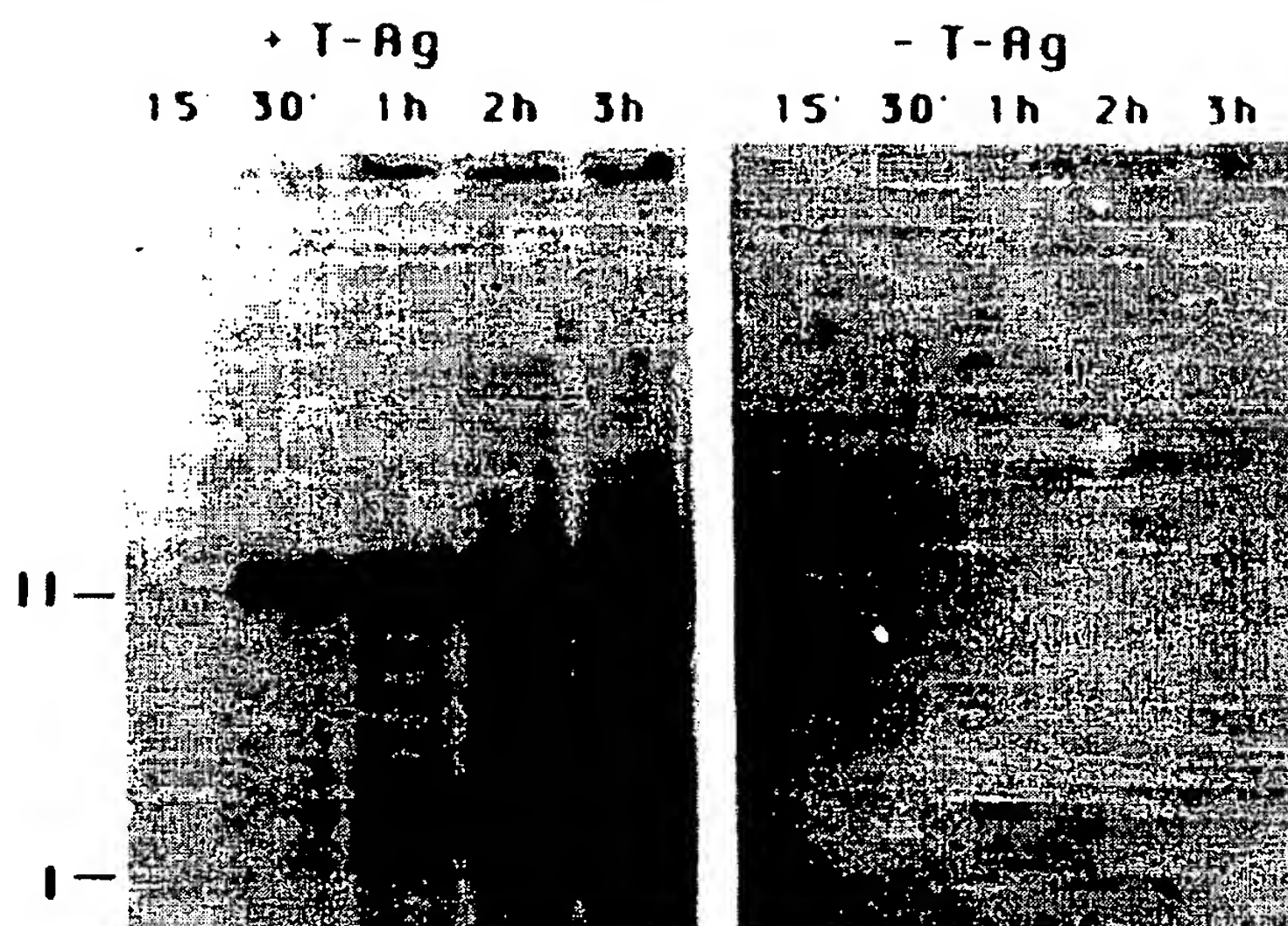


Fig. 5. Gel electrophoretic analysis of the product from the assay of in vitro SV40 DNA replication activity that was eluted from the Mono Q column. The assay for the replication of SV40 DNA in vitro was performed in the presence or absence of T-ag using 10 μ g protein from the 0.5 M KCl/buffer-C eluted peak

from the Mono Q column. Incubation was for the indicated times after which the product was isolated and electrophoresed on a 1.5% agarose gel. Details of the assay are given under Materials and Methods. Form I, supercoiled, closed circle DNA; Form II, relaxed, open circle DNA.

Sedimentation and Electrophoretic Analysis of the Purified Enzyme Complex

Sedimentation analysis on a 10 to 35% glycerol gradient of the eluted peak from Mono Q containing the activities of enzymes for DNA synthesis and SV40 replication activity showed that these activities remain associated as a 21 S complex during Mono Q FPLC (Fig. 6A,B). These activities sedimented coincidentally as a 21 S peak as did also the DNA-dependent ATPase, DNA ligase, 3' to 5' exonuclease, primase, RNase H, and topoisomerase I activities that were recovered with the 0.5 M KCl eluted peak from the Mono Q column (data not shown). The 21 S enzyme complex, however, did not cosediment with the bulk of the protein that was loaded onto the glycerol gradient but only resided with the leading shoulder of the protein profile in the gradient (compare Fig. 6A,B with 6C). The enzyme and SV40 DNA replication activities cosedimented as a 21 S complex when the sedimentation analysis was performed in presence of 0.15 M or 0.5 M KCl (data not shown). SDS PAGE analysis of fractions throughout the protein profile in the gradient showed a complex gel pattern (Fig. 6D). Gel analysis of fractions within the peak of DNA polymerase α and SV40 DNA repli-

cation activities showed approximately 25 (fractions 7–11) to 30 (fraction 13) polypeptide polypeptide bands, while fractions within the slower sedimenting protein peak (i.e., fractions 15–17 in Fig. 6C), which lacked DNA polymerase and SV40 DNA replication activity, showed 35 to 40 polypeptide bands. Some of the polypeptide bands such as the 180, 120, 110, 45–47, 33, and 27 kDA polypeptides were present only with fractions within the peak containing the enzyme and SV40 replication activities. However, a number of polypeptide bands, especially those in the of 50 to 20 kDA range, were present in fractions throughout the protein profile of the gradient. The intensity of staining of some of these bands, however, differed between the two protein peaks in the gradient.

Sedimentation analysis of the in vitro SV40 DNA replication activity in the crude NE/S-3 fraction showed that the activity with this fraction, as well as the activity associated with Mono Q FPLC purified enzyme complex, sedimented as a 21 S peak (Fig. 7). These results suggest that the 21 S enzyme complex exists in crude cell extracts and that the complex remains intact during the course of its purification including the Mono Q FPLC step.

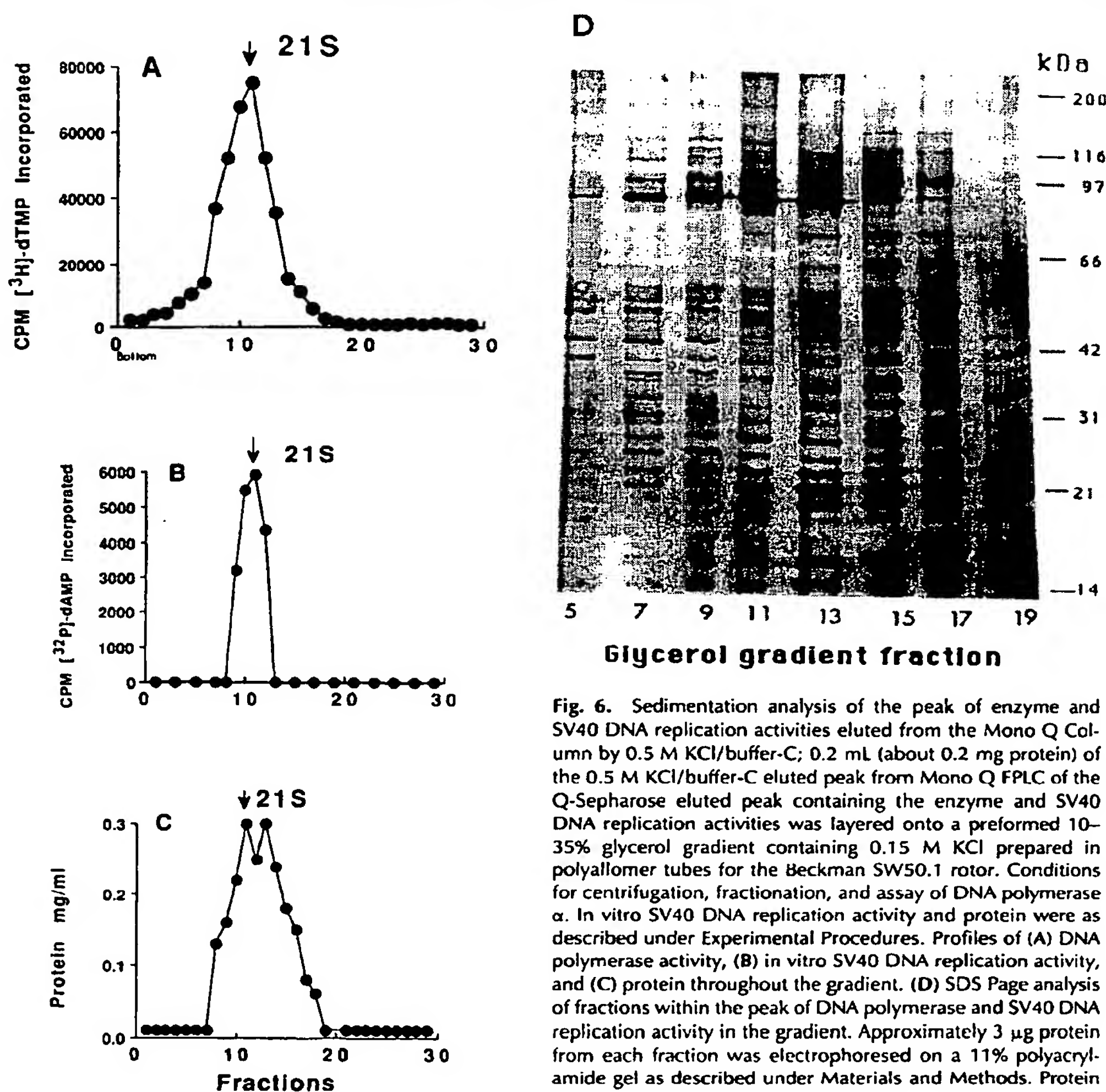


Fig. 6. Sedimentation analysis of the peak of enzyme and SV40 DNA replication activities eluted from the Mono Q Column by 0.5 M KCl/buffer-C; 0.2 mL (about 0.2 mg protein) of the 0.5 M KCl/buffer-C eluted peak from Mono Q FPLC of the Q-Sepharose eluted peak containing the enzyme and SV40 DNA replication activities was layered onto a preformed 10–35% glycerol gradient containing 0.15 M KCl prepared in polyallomer tubes for the Beckman SW50.1 rotor. Conditions for centrifugation, fractionation, and assay of DNA polymerase α . In vitro SV40 DNA replication activity and protein were as described under Experimental Procedures. Profiles of (A) DNA polymerase activity, (B) in vitro SV40 DNA replication activity, and (C) protein throughout the gradient. (D) SDS Page analysis of fractions within the peak of DNA polymerase and SV40 DNA replication activity in the gradient. Approximately 3 μ g protein from each fraction was electrophoresed on a 11% polyacrylamide gel as described under Materials and Methods. Protein bands were visualized by silver staining.

Immunological Analysis of the DNA Polymerase Associated With the Purified 21 S Enzyme Complex

The results from previous studies showed that the 21 S complex of enzymes for DNA synthesis resides exclusively with the sedimentable P-4 subfraction of the NE/S-3 and is absent from the nonsedimentable S-4 subfraction [Baril et al., 1988; Hickey et al., 1988]. It was also shown that the previously described 640 kDa multiprotein DNA polymerase α -primase complex [Vishwanatha et al., 1986] resides exclusively with P-4, even though the S-4 does contain

activities of DNA polymerase α , primase, and other enzymes for DNA synthesis [Hickey et al., 1988].

Immunoblot analysis with monoclonal antibody SJK 237 [Tanaka et al., 1982] to human DNA polymerase α was performed on the purified 21 S enzyme complex to determine if the multiprotein 640 kDa DNA polymerase α -primase complex is associated with the 21 S enzyme complex during the course of its purification. Electrophoresis of the crude cytoplasmic extract (S-3) and the Mono Q FPLC purified 21 S complex was performed under nondenaturing

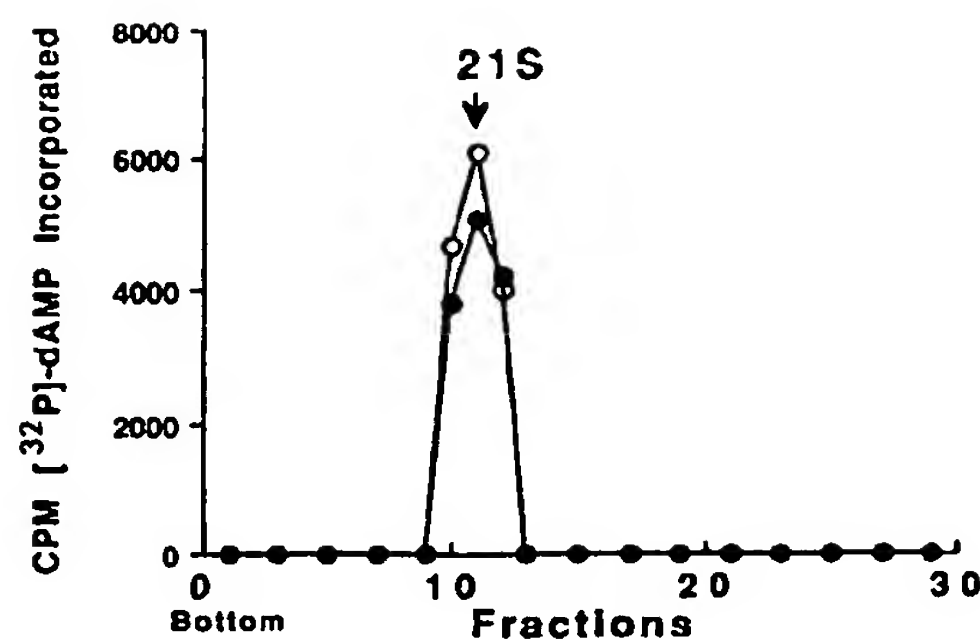


Fig. 7. Sedimentation profile of the in vitro SV40 DNA replication activity in the crude NE/S-3 and the 0.5 M KCl/buffer-C eluted peak from Mono Q FPLC of the PS. Approximately 0.2 mg (0.2 mL) of the NE/S-3 and the 0.5 M KCl/buffer-C eluted fraction from Mono Q FPLC of the PS were loaded onto preformed 10 to 35% glycerol gradients in buffer-A containing 0.15 M KCl. The conditions for centrifugation, fractionation of the gradient, and assay of SV40 DNA replication activity were as described under Materials and Methods. Following the centrifugation and assay, SDS PAGE analysis was performed on aliquots of fractions containing the peak of SV40 DNA replication activity from the respective gradients. In Vitro SV40 DNA replication activity in fractions throughout the gradient. Open circles, fractions from gradient centrifugation of the NE/S-3; Closed circles, fractions from gradient centrifugation of the 0.5 M KCl/buffer-C eluate from the Mono Q column.

conditions that are known to preserve the structure of the 640 kDa multiprotein DNA polymerase α -primase complex [Lamothe et al., 1981]. The results from immunoblots of the crude S-3 and the Mono Q FPLC purified 21 S enzyme complex both showed a predominant band of 640 kDa, as well as a faint band of 180 kDa (Fig. 8A). The latter is attributable to some dissociated DNA polymerase α catalytic polypeptide [Vishwanatha et al., 1986]. Association of the 640 kDa multiprotein DNA polymerase α -primase complex with the Mono Q FPLC purified 21 S complex was corroborated by chromatographic fractionation of the purified 21 S enzyme complex under the conditions developed for the isolation of the intact 640 kDa multiprotein polymerase α -primase complex [Vishwanatha et al., 1986]. Essentially all (more than 95%) of the DNA polymerase activity associated with purified 21 S enzyme complex was recovered as the 640 kDa multiprotein DNA polymerase α -primase complex (Li and Baril, unpublished data).

The DNA polymerase activity, assayed under optimal conditions for the measurement of DNA polymerase α and δ activities, and the SV40 DNA replication activity associated with the pu-

rified 21 S enzyme complex were both completely neutralized by SJK 132-20 monoclonal antibody to human polymerase α (Fig. 8B). These results suggest that the predominant DNA polymerase activity associated with the 21 S enzyme complex is DNA polymerase α and corroborate earlier evidence showing that its activity is essential for initiating SV40 DNA replication in vitro [Ishimi et al., 1988; Wold et al., 1989].

Comparison of the Nuclear Localization DNA Polymerases α , β , and DNA Ligase I During S-Phase

Nuclear localization of DNA polymerase α in mammalian cells was demonstrated by subcellular fractionation in nonaqueous buffers [Foster and Gurney, 1976] and immunofluorescent staining [Stokke et al., 1991]. The tight nuclear binding of DNA polymerase α was shown to be specific for cells in S-phase of the cell cycle [Stokke et al., 1991]. Nuclear localization of bovine kidney epithelial cell (MDBK) DNA ligase I was also shown by immunofluorescent staining [Lasko et al., 1990].

Since both DNA polymerase α and DNA ligase I are associated with the 21 S enzyme complex that functions in T-Ag-dependent SV40 DNA replication in vitro, we compared their nuclear localizations by immunofluorescent staining during S phase in synchronized HeLa cells. The nuclear localizations were compared with that of DNA polymerase β , an enzyme that is believed to function primarily in DNA repair [Lehman and Kaguni, 1989; Fry and Loeb, 1986].

For cells in S-phase of the cell cycle, the intranuclear immunofluorescent staining with antibodies to DNA polymerase α and DNA ligase I was localized with granular-like structures in the nucleoplasm and along the nuclear envelope (Fig. 9A,B). This immunofluorescent localization was observed throughout S-phase. For cells at the G1/S boundary, immunofluorescent staining was localized with granular-like structures distributed throughout the nucleoplasm. At the S/G2 boundary the immunofluorescent staining was localized predominately with granular-like structures adjacent to the nuclear envelope (data not shown). In contrast, the intranuclear immunofluorescent staining of cells in S-phase with antibody to DNA polymerase β was diffusely distributed throughout the nucleoplasm (Fig. 9C). This was observed for nuclei of cells at the G1/S boundary and throughout S-phase of the

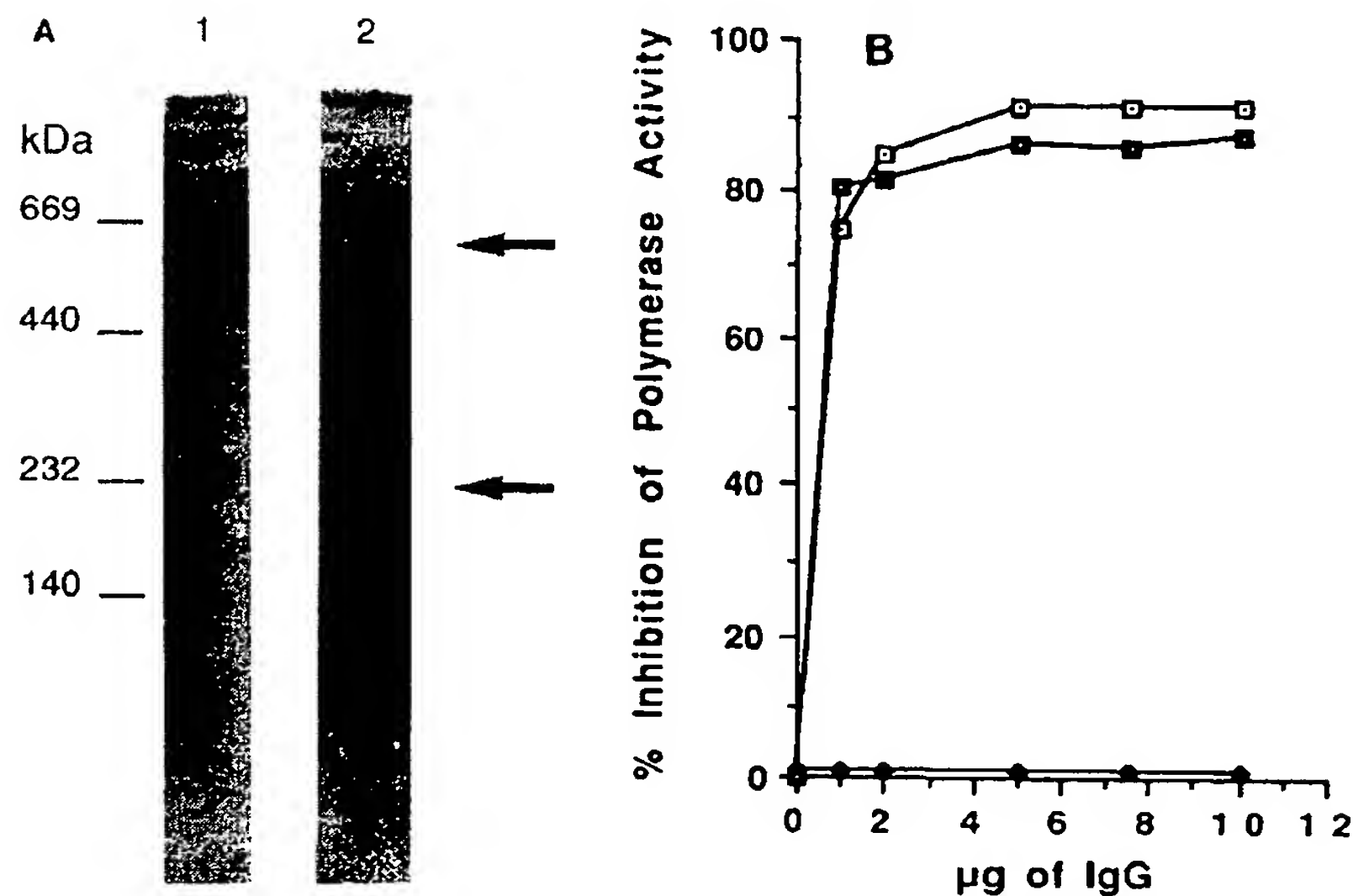


Fig. 8. Immunological analysis of the DNA polymerase associated with the purified 21 S enzyme complex (A). Immunoblots of DNA polymerase α in the crude NE/S-3 and the Mono Q FPLC purified 21 S enzyme complex electrophoresed under nondenaturing conditions on 4% polyacrylamide gels and immunoblotted with monoclonal antibody SJK 237-71 to human DNA polymerase α . The conditions for electrophoresis under nondenaturing conditions, electrotransfer of proteins and immunoblotting are described under Materials and Methods. A: Immunoblot of DNA polymerase α in the crude NE/S-3 (lane 1) and the Mono Q FPLC purified 21 S enzyme complex (lane 2). Native protein markers were; thyroglobulin (669 kDa), apoferritin (440 kDa), catalase (232 kDa), and lactate dehydrogenase (140 kDa). Arrows indicate the position of migration in the gel of the 640 kDa multiprotein DNA polymerase α -primase complex and the 183 kDa polymerase α catalytic subunit. B: Neutralization of the DNA polymerase activity associated with the

Mono Q FPLC purified 21 S enzyme complex by monoclonal antibody SJK 132-20 to human DNA polymerase α . Aliquots (4 μ g protein) of the purified 21 S complex were preincubated with the designated amounts of SJK 132-20 or preimmune mouse control IgGs at 4°C for 1 h prior to initiating the DNA polymerase assay by addition of template and other components for the assay of DNA polymerase α [Lamothe et al., 1981] or DNA polymerase δ [Tan et al., 1986] activity. Incubation was continued at 35°C for 30 min and the reaction was terminated and the incorporated radioactivity measured according to published procedures [Lamothe et al., 1981; Vishwanatha et al., 1986; Tan et al., 1986]. The activity of DNA polymerase α (no inhibition) in the assay was 3 units and the activity of DNA polymerase δ was 1.2 units. Open squares, DNA polymerase α assay; closed squares, DNA polymerase δ assay; closed triangles, DNA polymerase α assay with preimmune mouse IgG (control).

cell cycle. There was no distinct intranuclear localization of the immunofluorescent staining with granular-like structures as observed with antibodies to DNA polymerase α and DNA ligase I.

DISCUSSION

There is increasing evidence for the existence in eukaryotic cells of megadalton, multiprotein complexes that participate in macromolecular metabolism. These multisubunit structures are sedimentable subribosomal particles that vary in size from 20 S to 50 S. As in the case of ribosomes, the multiprotein spliceosome complexes [Green, 1986; Sharp, 1987] and signal recognition particles [Walter and Blobel, 1981] are composed of RNA and protein. Other multi-

protein complexes, such as proteasomes [Orlowski, 1990; Rechsteiner et al., 1993] and aminoacyl tRNA synthetase complexes [Orlowski, 1990; Negrutskii and Deutscher, 1992], are proteinaceous and have associated multicatalytic activities. There have also been reports that enzymes involved in DNA replication in eukaryotic cells exist as megadalton, multiprotein complexes [Tubo and Berezney, 1987a; Jackson and Cook, 1986a; Jazwinski and Edelman, 1984; Reddy and Pardee, 1980].

The recent advent of a cell free system for SV40 DNA replication in vitro now provides a means for the analysis of the DNA synthesizing machinery in primate cells [Challberg and Kelly, 1989; Stillman, 1989]. The results from fractionation and reconstitution studies using human

cell extracts indicate that in addition to the viral encoded T-ag a minimum of seven to ten cellular proteins, or a total of 17 to 22 polypeptides, are involved in the initiation of SV40 DNA replication in vitro [Weinberg et al., 1990; Tsurimoto and Stillman, 1990; Hurwitz et al., 1990]. These include DNA polymerases α and δ , primase, proliferating cell nuclear antigen (PCNA), a polymerase ATPase accessory protein complex (RF-

C), a DNA binding protein complex (RP-A), and topoisomerase I and DNA ligase [Challberg and Kelly, 1989; Stillman, 1989; Wold et al., 1989; Tsurimoto and Stillman, 1990; Weinberg et al., 1990; Hurwitz et al., 1990].

In this paper we report on the purification of the 21 S complex of enzymes for DNA synthesis that exists in crude HeLa cell extracts. All of the in vitro SV40 DNA replication activity in the crude NE/S-3, as well as in intermediate steps during the purification, reside exclusively with the 21 S enzyme complex. SDS PAGE analysis of the purified 21 S complex showed the presence of approximately 30 polypeptides of molecular mass in the range of 300 to 15 kDa. We can account for 18 of the polypeptides on the basis of enzymes and other proteins that have been purified from the enzyme complex. It is not yet known if all 30 polypeptides represent bona fide components of the purified enzyme complex. This number may not be unreasonable, however, considering the number of proteins and polypeptides that are reported from in vitro reconstitution studies to be required for initiation of SV40 DNA replication [Challberg and Kelly, 1989; Stillman, 1989; Wold et al., 1989; Tsurimoto and Stillman, 1990; Weinberg et al., 1990; Hurwitz et al., 1990]. Some of the enzymes and proteins associated with the crude and purified 21 S complex (e.g., DNA polymerase α -primase, topoisomerase I, DNA ligase, PCNA, RNase-H, etc.) have also been reported from in vitro reconstitution studies to be required for SV40 DNA replication. Taken together, these results support a function of the 21 S enzyme complex in the initiation of SV40

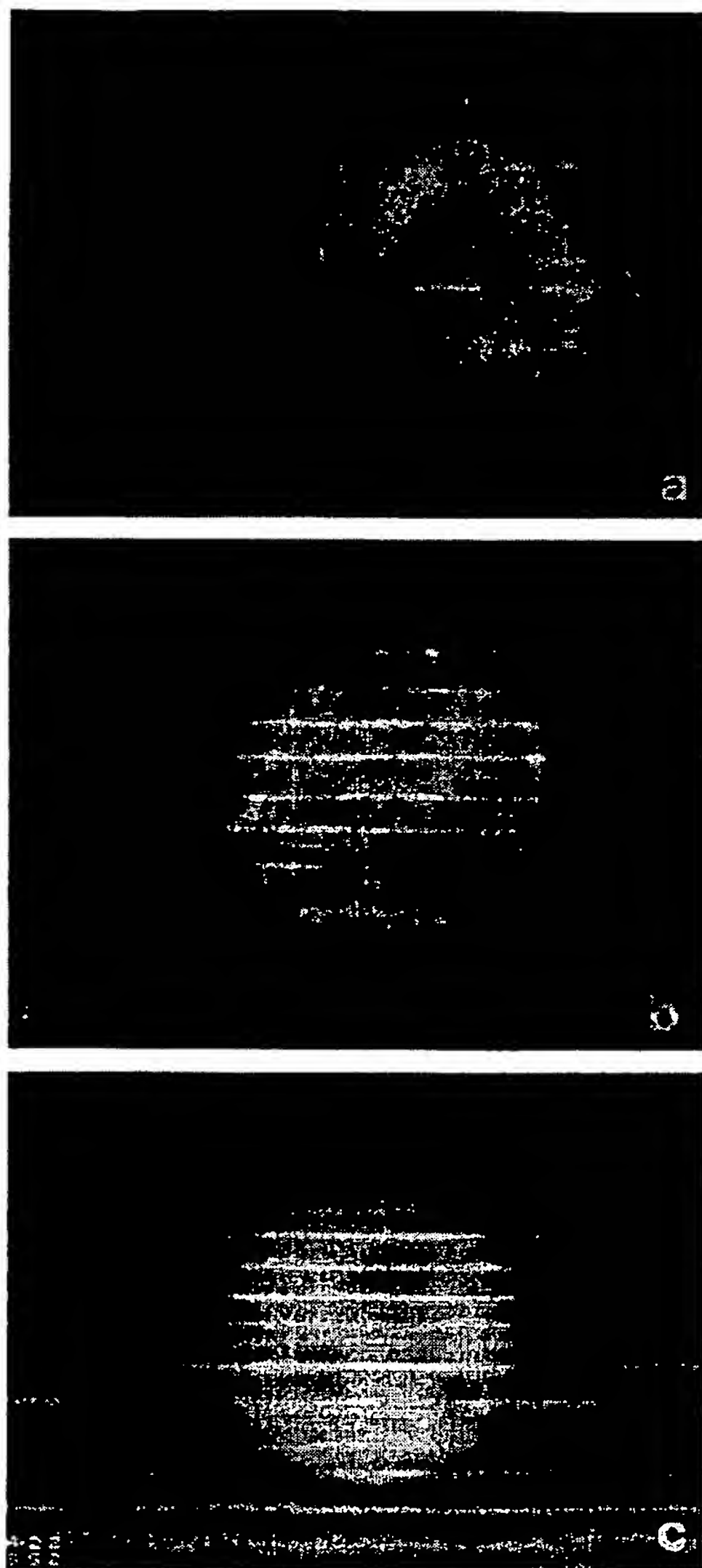


Fig. 9. Intranuclear immunolocalization of DNA polymerases α , β , and DNA ligase I in HeLa cells in S-phase of the cell cycle. HeLa S₃ cells in suspension culture were synchronized by the double thymidine block technique and synchronization was monitored by 10 min pulses of ³H-thymidine following release from the second thymidine block [Chiu and Baril, 1975]. Cells were harvested at various times following the release from the second thymidine block, i.e., G1/S boundary; early, mid, and late S-phase, S/G2 boundary of the cell cycle. The procedures for immunofluorescent staining and imaging are described under Methods. a: Immunofluorescent staining pattern in the nucleus of cells in mid-S phase using monoclonal antibody (SJK 237-71) to human DNA polymerase α [Tanaka et al., 1982]. b: Immunofluorescent staining pattern in the nucleus of cells in mid S phase using rabbit anti-calf thymus DNA ligase I IgG that cross-reacts with human DNA ligase I [Tomkinson et al., 1990]. c: Immunofluorescent staining pattern in the nucleus of cells in mid-S phase using monoclonal antibody to recombinant DNA polymerase β .

DNA replication *in vitro*. Its existence in crude HeLa cell extracts suggest that it may be a part of the chromosomal DNA synthesizing machinery in human cells.

The results from biochemical and immunological analyses indicate that the major DNA polymerase associated with the purified 21 S complex is DNA polymerase α . Recent fractionation/reconstitution studies indicate that the DNA polymerase α -primase complex is required for initiation and for lagging strand synthesis in SV40 DNA replication *in vitro* [Masumoto et al., 1990]. DNA polymerase δ is also believed to function in leading strand synthesis once initiation by DNA polymerase α has occurred [Fairman and Stillman, 1988; Stillman, 1989; Hurwitz et al., 1990]. Our results are not necessarily in conflict with this proposal. Hurwitz and co-workers [Ishimi et al., 1988; Hurwitz et al., 1990] reported that both a monopolymerase (i.e., polymerase α -primase only) and dipolymerase (i.e., polymerase α -primase and polymerase δ) system reconstituted from human cell extracts can function in SV40 DNA replication *in vitro*. It has also been shown that once initiation of SV40 DNA replication *in vitro* by DNA polymerase α occurs, any DNA polymerase holoenzyme, including those from prokaryotes, can complete leading strand DNA synthesis [Tsurimoto and Stillman, 1990; Matsumoto et al., 1990]. Thus, it is quite possible that the 21 S complex functions as a monopolymerase system in SV40 DNA replication *in vitro*.

DNA polymerase α and DNA ligase I are both predominately associated with the 21 S enzyme complex. There is good evidence that both of these enzymes function in DNA replication [Fry and Loeb, 1987; Lehman and Kaguni, 1989; Tomkinson et al., 1990]. DNA polymerase β , on the other hand, appears to function primarily in DNA repair and is not associated with the 21 S enzyme complex for DNA synthesis. Our results from immunofluorescent staining using antibodies to DNA polymerase α and DNA ligase indicate that these enzymes are localized solely with granular-like structures during G1 and S phases of the cell cycle. Immunofluorescent localization of DNA polymerase β , however, showed a diffuse distribution within the nucleoplasm throughout the entire cell cycle. Other investigators have also observed intranuclear immunolocalization of DNA polymerase α [Yamamoto et al., 1984; Stokke et al., 1990; Hozak et al., 1993], DNA ligase I [Lasko et al., 1990], and a population of

PCNA molecules [Bravo and Macdonald-Bravo, 1987; Kill et al., 1991] with granular-like structures during S phase of the mammalian cell cycle.

The importance of the nuclear architecture in the regulation of RNA [Spector, 1990; Fu and Maniatis, 1990; Carter et al., 1991] and DNA synthesis [Berezney and Coffey, 1975; Blow and Laskey, 1988; Cook, 1991; Jackson, 1991] is now being recognized. Intranuclear localization of pre-messenger RNA to discrete sites in mammalian cells was shown by fluorescence hybridization, immunofluorescence, and digital imaging microscopy [Xing and Lawrence, 1991; Wang et al., 1991; Carter et al., 1993]. These foci have recently been reported to reside with the nuclear matrix at sites that contain the mRNA-splicing machinery [Spector, 1991; Carter et al., 1993; Xing et al., 1993]. Limited intranuclear sites for DNA replication and association of enzymes for DNA replication have also been reported to reside with the nuclear matrix at least through part of the cell cycle [Berezney and Coffey, 1975; Jackson and Cook, 1986b; Tubo and Berezney, 1987b; Nakayasu and Berezney, 1989; Mills et al., 1989; Hozak et al., 1993]. It is possible that the 21 S enzyme complex is related to such a structure.

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